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Dissertação

Mestrado em Biologia Evolutiva e do Desenvolvimento

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Abstract

Antibiotic resistance in pathogenic bacteria is an increasing concern in health programs, and a number of alternatives are currently under investigation. One promising alternative is phage therapy, the use of specific bacteriophages to control or eradicate pathogenic bacteria. Despite having many advantages, the use of phages still presents some limitations and requires experimental investigation. Phage therapy benefits from the abilities of phages to multiply *in situ* and evolve, but bacteria can also evolve resistance to phages, in a process called antagonistic coevolution. Through the coevolution and evolution of wild type and mutator strains of *Pseudomonas fluorescens* and its lytic phage ϕ2, this study sought to determine how the nature of selection affects phage killing efficiency. Phages were sequentially transferred either with bacteria (coevolution) or passaged on the ancestral bacteria (evolution). The efficiency of phages from three time points (transfers 1, 4 and 8) was estimated by plating on ancestral and allopatric coevolved bacteria. The results indicate that the coevolution treatment leads to a greater increase in phage efficiency than the evolution treatment. This is specially seen when phage populations are confronted with mutator coevolved bacteria, which may be due to a specialization cost to the evolved phage populations. Wild type coevolved bacteria were resistant to all allopatric phages, whether from the past, present or future. Phage infectivity to ancestral bacteria peaked early in the transfer regime. Further studies are necessary to confirm these results and should consider the effects of phage dose.

Key words: coevolution, evolution, *Pseudomonas fluorescens*, phage therapy, mutator bacteria

Resumo

O alastramento de infecções hospitalares com bactérias resistentes a antibióticos têm vindo a aumentar nos últimos anos. A evolução de bactérias resistentes a antibióticos tem sido acelerada, em parte, devido ao excessivo uso destas substâncias, durante mais de meio século, não só no tratamento médico de pacientes, mas também na criação de animais para consumo humano. Também a evolução de resistência simultânea a múltiplos antibióticos tem vindo a aumentar em bactérias patogénicas graças à existência de elevadas taxas de mutação e à ocorrência de transmissão horizontal de genes. Por outro lado, os custos da resistência a antibióticos tendem a diminuir rapidamente no curso da evolução, o que faz com que a simples suspenção temporária do uso de um dado antibiótico não seja suficiente para eliminar os genótipos resistentes. Também uma diminuição na produção de novos antibióticos tem vindo a tornar clara a necessidade de criação de alternativas à simples administração de um antibiótico. Algumas alternativas incluem a alternância do uso de mais do que uma classe de antibióticos ao longo de vários meses ou anos e o uso simultâneo de diferentes antibióticos em diferentes pacientes no mesmo hospital (chamado "mixing") ou no mesmo paciente. Outros métodos incluem a utilização de hidrolases da parede celular bacteriana (enzimas que degradam o principal constituinte da parede celular bacteriana, os peptidoglicanos, levando à lise celular e podendo ter origem em células eucarióticas, bacterianas e em bacteriófagos) e de péptidos antimicrobiais (péptidos com grande actividade bactericida e também com origem em células eucarióticas, bacterianas e em bacteriófagos). Embora apresentem algumas características promissoras, como a baixa capacidade que as bactérias apresentam para evoluir resistência a algumas destas moléculas, elevados custos de produção e a sua potencial toxicidade fazem com que estes métodos ainda necessitem de aperfeiçoamento antes que possam ser aplicados.

Uma outra alternativa à utilização de antibióticos é a terapia fágica, que consiste na utilização de bacteriófagos (ou fagos) específicos para erradicar ou controlar bactérias patogénicas. Após a descoberta, no início do século 20, da capacidade dos fagos de matarem bactérias, esta técnica foi durante várias décadas desenvolvida e aplicada apenas nos países da ex-União Soviética. Apenas recentemente voltou a ser considerada nos países Ocidentais, dada a urgência em desenvolver alternativas à utilização de antibióticos. Os bacteriófagos têm várias vantagens face ao uso de antibióticos, entre as quais se podem destacar o facto de se multiplicarem exponecialmente no local da infecção; serem bastante específicos no seu hospedeiro, o que protege a flora normal do organismo do paciente e diminui o número de estirpes bacterianas que lhes poderão evoluir resistência; serem eficientes contra bactérias resistentes a antibióticos; e poderem ser combinados com outros fagos ou com antibióticos para aumentar a sua eficácia. No entanto, a terapia fágica

também apresenta algumas limitações, como o facto de os fagos utilizados para este fim terem de ser obrigatoriamente fagos líticos (que injectam o seu material genético dentro das células bacterianas e libertam a sua progenia através da indução da lise bacteriana); com uma baixa capacidade de transferir material genético entre células bacterianas; e, preferencialmente, com o seu genoma totalmente sequenciado (para evitar a presença de genes indesejados, como os que codificam proteínas tóxicas para os pacientes). A existência da várias limitações à aplicação da terapia fágica sublinha a importância da investigação nesta área, para garantir uma adequada selecção e formulação dos fagos utilizados.

A capacidade que os fagos têm de evoluir *in situ* pode também ser considerada como uma desvantagem, dado o facto de tornar o seu comportamento algo imprevisível. No entanto, esta característica está na base da sua capacidade de se adaptarem ao seu hospedeiro. Infelizmente, as bactérias têm também a capacidade de evoluir resistência aos fagos, originando um processo designado por coevolução antagonística entre um parasita e o seu hospedeiro: o hospedeiro evolui resistência ao seu parasita devido à selecção exercida pelo parasita e, reciprocamente, ocorre a evolução da infectividade do parasita, contrapondo assim os mecanismos de defesa do hospedeiro. Os microorganismos são um modelo que apresenta bastantes vantagens para o estudo da evolução e coevolução, como o facto de terem tempos de gerações curtos e serem fáceis de manter em grande número, o que lhes permite evoluirem rapidamente no decurso de uma experiência. Outra vantagem é o facto de poderem ser congelados indefenidamente, o que permite a comparação de diferentes pontos temporais do processo evolutivo.

Este estudo utilizou o sistema *Pseudomonas fluorescens* SBW25 e o seu fago lítico associado ϕ2 (onde já foi demonstrada a ocorrência de coevolução antagonística) para estudar o efeito da evolução e coevolução na capacidade dos fagos de matarem bactérias (um dos prerequesitos da terapia fágica). O seu objectivo é contribuir para o desenvolvimento de um protocolo experimental que aumente a eficiência dos fagos, melhorando assim o controlo de bactérias patogénicas. Estudos anteriores verificaram que estes diferentes tipos de selecção afectam diferentemente a virulência dos fagos. Nesta experiência, os fagos foram coevoluídos (transferidos sequencialmente em conjunto com as bactérias) ou evoluídos (transferidos sequencialmente para as bactérias ancestrais) com duas estirpes de *P. fluorescens*, wild-type (WT) e *mutS*- (mutS) (um "hypermutator"). A estirpe mutS SBW25 tem um knockout no gene *mutS*, o que lhe confere uma taxa de mutação aproximadamente 100 vezes superior à do WT (mutS tem uma taxa de mutação de *c* $5x10^{-5}$ mutações por par de bases, por geração). Esta estirpe foi utilizada devido ao facto de bactérias com elevadas taxas de mutação ("mutators") serem frequentemente encontradas em populações naturais e laboratoriais e estarem frequentemente associadas a infecções clínicas. Experiências

laboratoriais demonstraram também que estas bactérias têm uma maior probabilidade de evoluir e conservar resistência a antibióticos na presença de fagos.

Foram realizadas 8 transferências sequenciais (uma a cada 48h). As populações fágicas iniciais cresceram a partir de culturas de WT ou mutS ancestrais e foram estimadas através do seu plaqueamento em tapetes bacterianos (da mesma estirpe ancestral na qual cresceram) e da contagem no número de placas fágicas (PfUs). 24 microcosmos com fagos e bactérias foram preparados, cada um através da adição de *c* 10^4 partículas fágicas, vindas da população inicial, e de 60µl de bactérias crescidas overnight (*c* 10^7 células) a 6ml de meio Kings B (KB).

Em 12 destas populações, os fagos foram confrontados com bactérias WT e, nas outras 12 populações, com bactérias mutS. Metade das réplicas de cada tratamento foram coevoluídas, sendo que a cada passagem foram transferidos 60µl de cultura para novos microcosmos com 6ml de meio KB. Na outra metade das réplicas de cada tratamento (evolução), os fagos foram isolados a cada passagem, e 60µl de solução fágica, juntamente com 60µl da estirpe bacteriana ancestral correspondente crescida overnight, foram transferidos para um novo microcosmos com 6ml de meio KB. Foram guardados stocks a -80ºC.

Fagos de três pontos temporais (transferências 1, 4 e 8) foram isolados e todas as populações fágicas foram testadas em tapetes bacterianos de bactérias WT e mutS, quer ancestrais, quer previamente coevoluídas até à 4ª transferência (numa experiência preliminar, portanto as bactérias eram alopátricas aos fagos). A densidade de partículas fágicas que mataram bactérias (a eficiência fágica) foi estimada através da contagem de placas fágicas.

Os fagos coevoluídos mostraram-se mais eficientes do que os fagos evoluídos, embora exista a possibilidade de haver um efeito de dose (o aumento no número de fagos observado pode reflectir um aumento na eficiência de ataque por partícula fágica ou um aumento no número de fagos). Para corrigir um possível efeito de dose foram testados modelos estatísticos em que o número de fagos foi expresso em relação ao número de fagos obtido nas bactérias WT ancestrais. Foi demonstrado que as bactérias WT ancestrais não são sensíveis a todos os fagos, o que indica que o número de fagos pode ser subestimado nestas bactérias.

Todas as bactérias coevoluídas mostraram-se mais resistentes aos fagos que as ancestrais. As bactérias WT coevoluídas (até t4) mostraram-se totalmente resistentes a todas as populações de fagos (alopátricos), quer estes fossem coevoluídos ou evoluidos (em bactérias WT ou mutS), e quer fossem fagos do passado (t1), presente (t4) ou futuro (t8). Isto poderá estar relacionado com a aquisição de custos de resistência. Já as bactérias mutS coevoluídas (até t4) mostraram-se sucessivamente mais sensíveis as fagos t1, t4 e t8. Estas bactérias poderão ter uma desvantagem face

aos fagos (em comparação com as bactérias WT). Também as populações de fagos coevoluídos mostraram-se mais eficientes quando confrontadas com as bactérias coevoluídas do que as populações de fagos evoluídos, o que pode dever-se ao facto de os fagos evoluídos em bactérias ancestrais sofrerem um custo nas bactérias coevoluídas.

Todas as populações fágicas aumentaram em eficiência até à 8ª transferência face às bactérias mutS coevoluídas. No entanto, quando confrontados com as bactérias ancestrais, os fagos aumentaram maioritariamente a sua eficiência até t4, tendo-a mantido entre t4 e t8. Isto pode indicar que os fagos atingiram o seu pico de eficiência face às bactérias ancestrais após um curto número de transferências e que, no caso dos fagos coevoluídos, estes poderão sofrer custos de especialização. Devido ao potencial efeito de dose, mais estudos serão necessários para confirmar estes resultados.

Palavras-chave: coevolução, evolução, *Pseudomonas fluorescens*, terapia fágica, bactérias "mutator"

Index

Introduction

The appearance and spread of antibiotic resistant bacteria that cause nosocomial infections is a widely known problem (Wenzel & Edmond 2001, Pirnay et al. 2012, Rice 2008, Bonhoeffer et al. 1997). The evolution of bacterial resistance to antibiotics continues to increase due to the excessive use of antibiotics, not only in human medicine, but also in the breeding of animals for human consumption (Kümmerer & Henninger 2003, Kümmerer 2004). Also, multi-antibiotic resistance in pathogenic bacteria is increasing (McCormick et al. 2003, Shah et al. 2007). This is due not only to more than half a century of selection through intensive antibiotic use, but also to the great genetic flexibility in certain bacteria, due to, for example, high mutation rates and the horizontal transfer of genes (Mazel & Davies 1999).

Since evolutionary changes in bacteria can decrease the cost of antibiotic resistance over short periods of time (e.g. by compensatory beneficial mutations or positive epistasis), resistant genotypes do not disappear if particular antibiotics are only temporarily removed from healthcare (Lenski 1998, Maisnier-Patin et al. 2002, Trindade et al. 2009, Sousa et al. 2011, Silva et al. 2011). The decline in the supply of new antibiotics (Donadio et al. 2010) has led to the study of several alternatives to simple antibiotic administration. These include alternating the use of more than one class of antibiotics over large time scales (months or years), with the intention of decreasing, or at least stabilizing, the evolution of resistance to specific antibiotics (Kollef 2001, McGowan 1986). However, there are some questions regarding the efficacy of this method (Toltzis et al. 2002, Bergstrom et al. 2004). Other methods, like the simultaneous use of different antibiotics on different patients in the same hospital (mixing) or in the same patient may be more efficient (Bergstrom et al. 2004, Bonhoeffer et al. 1997).

Additional methods to control bacterial pathogens include the application of bacterial cell wall hydrolases (Masschalck & Michiels 2003, Parisien et al. 2008) and antimicrobial peptides (Parisien et al. 2008). Bacterial cell wall hydrolases are enzymes that degrade the major component of the bacterial cell wall, peptidoglycans, leading to the cell lysis (Masschalck & Michiels 2003, Parisien et al. 2008). These can be derived from eukaryotic cells (lysozymes), bacterial cells (autolysins) and bacteriophages (virolysins or endolysins) (Parisien et al. 2008). These enzymes have promising characteristics such as their efficiency against antibiotic-resistant bacteria, and their being well known and safe (Parisien et al. 2008). Among these, virolysins are the most promising, since bacteria appear incapable of evolving resistance to them (Loeffler et al. 2001, Schuch et al. 2002). The restrictions to Gram positive bacteria of most bacterial cell wall hydrolases and their high production costs are their biggest disadvantages (Fischetti 2010, Parisien et al. 2008).

Antimicrobial peptides are naturally occurring peptides, with various structures and functionalities (Parisien et al. 2008). Again, these can be derived from eukaryotic cells (Straus & Hancock 2006), bacteria (bacteriocins) (Kirkup Jr. 2006) and bacteriophages (Parisien et al. 2008). Their advantages include a potent bactericidal activity, low levels of induced resistance and considerable potential due to their variety of structures and functionalities. However, none of these peptides has been yet approved for medical use. Some of the major obstacles to their application are their toxicity and their high costs of development and manufacturing. More research is needed in this area (Parisien et al. 2008).

Another alternative is phage therapy. Phage therapy is the use of specific bacteriophages to eradicate or control pathogenic bacteria (Abedon 2009). After the discovery of the phage's potential to kill bacteria in the beginning of the 20th century, phage therapy was mostly developed and applied in countries of the former Soviet Union. Due to both the discovery of antibiotics in the 1940s (which are easier to apply) and the poor communication between these countries and Western society, only recently has research on phage therapy been taken seriously in the West (Summers 2001, Chanishvili et al. 2001, Dublanchet & Patey 2011). Even though their therapeutic use has been neglected in the West, bacteriophages continued to be used in fundamental research, especially in molecular biology (Dublanchet & Patey 2011). Nowadays, given the urgency of finding alternatives to antibiotics, they are again being studied with a therapeutic objective in animal models (Barrow et al. 1998, Debarbieux et al. 2010, Smith et al. 1987, Smith et al. 1987b, Smith & Huggins 1982, Morello et al. 2011) and in humans (Wright et al. 2009).

Bacteriophages have several advantages over the antibiotic treatment of bacterial infections. Bacteriophages specific to target bacteria (1) grow exponentially in number at the site of infection (Carlton 1999), (2) only minimally disrupt normal flora, because of their specificity, which also narrows their potential to induce resistance, since phage limit the number of bacterial types (strains) that are being selected for specific resistance mechanisms (Hyman & Abedon 2010, Kutateladze & Adamia 2010, Carlton 1999), (3) may be effective against antibiotic-resistant bacteria (Carlton 1999), (4) can be combined with antibiotics (Carlton 1999, Zhang & Buckling 2012, Escobar-Páramo et al. 2012) or other phages, increasing their efficacy (McVay et al. 2007, Tanji et al. 2005, Hall et al. 2012), (5) can be applied in various forms (e.g., creams, liquids, aerosols, etc) (Górski et al. 2007) and (6) can even disrupt bacterial biofilms (Abedon 2011), amongst several other advantages (Loc-Carrillo & Abedon 2011). Secondary (non-medical) advantages include their low environmental impact, since their main constituents are nucleic acid and proteins (Abedon 2010) ; that they can be rapidly inactivated (Loc-Carrillo & Abedon 2011) ; and their low production costs (Skurnik et al. 2007, Loc-Carrillo & Abedon 2011).

Despite these advantages, phage therapy can also present some limitations. For example, not all phages can be used as therapeutic agents: phages have to be (1) obligately lytic (phages that inject their genetic material inside the bacteria to replicate, leading to bacterial lysis and phage progeny release into the environment), so that they effectively kill the bacterial hosts (Lenski & Levin 1985), (2) stable under accessible storage conditions, (3) have a low ability to transfer bacterial genes between bacteria, and (4) preferably fully sequenced to avoid the presence of undesirable genes, like those coding for proteins that could be toxic for patients (Skurnik et al. 2007, Loc-Carrillo & Abedon 2011). Phages can also interact with the patient's immune system (Loc-Carrillo & Abedon 2011), even though some studies indicate that this may not be a concern for phage therapy (Kutateladze & Adamia 2010, Carlton 1999). This underlies the importance of research in assuring proper phage selection and formulation, so as to prevent these potential drawbacks of phage therapy.

Moreover, that phages can evolve *in situ* may be seen as a disadvantage, since it makes their behavior more difficult to predict (Loc-Carrillo & Abedon 2011). On the other hand, evolution is the basis for their ability to adapt to their host. Unfortunately, bacteria can also evolve resistance to phage, potentially giving rise to antagonistic coevolution. Antagonistic coevolution between a parasite and its host can be defined as the host's evolution of resistance based on selection exerted by the parasite and the evolution of the latter's exploitation (infection, growth, proliferation), thereby (possibly temporarily) countering host resistance (Thompson 1994). This process is thought to have an important influence on the evolution of parasite virulence (Bull 1994, Ebert & Hamilton 1996, Ebert & Bull 2003, Alizon et al. 2009), and is considered to be potentially one of the reasons that makes it advantageous to produce genetically variable progeny through sexual reproduction (Ebert & Hamilton 1996, West et al. 1999, Agrawal 2006). Microorganisms have numerous advantages for studying evolution and coevolution, such as short generation times and ease to maintain in large populations, which allows for a rapid evolutionary change during the course of an experiment. Also the fact that they can be easily stored (frozen) indefinitely allows to compare different time points in the evolutionary process and to retrieve historical populations to conduct new experiments and assays (Lenski et al. 1991, Bohannan & Lenski 2000).

In this study, the effect of evolution and coevolution on the ability of phages to kill bacteria, which would be one requirement for effective phage therapy, was analyzed. This subject has been addressed previously by Poullain et al. 2008, who verified that these different types of selection (evolution versus coevolution) would differently affect phage virulence. The system *Pseudomonas fluorescens* SBW25 and its lytic phage ϕ2, previously shown to antagonistically coevolve, was used (e.g. Buckling & Rainey 2002, Poullain et al. 2008, Paterson et al. 2010). The phages were coevolved

(sequentially transferred with bacteria) or evolved (repeatedly transferred to the ancestral bacteria) with two strains of *P. fluorescens*, WT and mutS (a hypermutator) (Rainey and Bailey 1996, Pal et al. 2007). The mutS strain was used because bacteria with high mutation rates (mutators) are often found in natural (LeClerc et al. 1996, Matic et al. 1997) and laboratory (Sniegowski et al. 1997, Giraud et al. 2001; Pal et al. 2007) populations, and are frequently associated with clinical infections (Oliver et al. 2000, Denamur et al. 2002). Mutator bacteria could pose a problem in hospital settings, because laboratory experiments have shown that they are more likely to evolve and conserve resistance to antibiotics in the presence of bacteriophages (Escobar-Páramo et al. 2012; Zhang & Buckling 2012). Phage's efficiency was tested on WT and mutS bacteria, both ancestral and previously coevolved with phage. The objective of this study is to contribute to the development of an experimental protocol that increases phage efficiency, with the aim of controlling bacterial pathogens.

Materials and Methods

System:

The gram-negative bacterium *Pseudomonas fluorescens* SBW25 and its lytic phage ϕ2, with which it has been shown to antagonistically coevolve, were used (Buckling and Rainey 2002, Poullain et al. 2008, Paterson et al. 2010).

The strains used were the wild-type (WT) strain (isolated from a sugar beet leaf and its genome fully sequenced by Rainey & Bailey 1996) and an isogenic hypermutator *mutS*- strain (mutS), which is a SBW25 *mutS* knockout mutant with an approximately 100-fold higher mutation rate (while the WT has a mutation rate of *c* $5x10^{-7}$ per base pair, per generation, the mutS has a mutation rate of *c* $5x10^{-5}$ per base pair, per generation) (Pal et al. 2007).

Culture Conditions:

The same culture conditions as Poullain et al. 2008 were employed: "*All populations were selected in batch culture in 6 mL Kings B (KB: 10 g glycerol, 20 g proteose peptone No. 2, 1.5 g magnesium sulphate, 1.5 g potassium phosphate, 1 L Millipore water) aerated microcosms, shaken 1 min every 30 min at 28 ◦C at 200 rpm in a rotating agitator (Brockhurst et al. 2003). Media used in plating bacteria and phage consisted of KB agar (liquid KB medium supplemented with 12 g bacteriological agar*."

Selection Experiment (see Table 1 for description of symbols):

Phages were either Coevolved (C) or Evolved (E) with WT (WT C_p and WT E_p, respectively) or with mutS bacteria (mutS C_p and mutS E_p, respectively) for 8 sequential transfers.

Bacterial populations were grown from inoculum introduced with a sterile loop of naive, ancestral WT or mutS *P. fluorescens* in 6ml of KB (in a 30 ml plastic tube). Cultures were grown overnight at 28ºC with constant rotational agitation at 200 rpm.

Phage populations were grown in overnight cultures of either WT or mutS naive strains, agitated 200 rpm 1 min. every 30 min. at 28ºC. After 24 hours, bacteria were selectively killed with 10% chloroform and centrifuged for 4 minutes at 13000 rpm to isolate phage in the supernatant. Phage population densities were estimated by plating onto bacterial lawns of the same naive ancestral strain with which they were grown, and counting PfUs (Curtin & Donlan 2006, Singh et al. 2009).

24 phage-bacteria microcosms were prepared by adding to each *c* 10^4 phage particles, from a master population, and 60 µl of exponentially growing bacteria (*c* 10^7 cells) to 6 ml of KB. Microcosms were rotationally agitated 200 rpm 1 min. every 30 min. at 28ºC.

In 12 of these populations, phages were confronted with WT bacteria and, in the other 12, phages were confronted with mutS bacteria. One-half of the replicates for each treatment were coevolved by transferring 8 times (one every two days) 60 µl of culture to fresh microcosms with 6 ml of KB. For the remaining half of the replicates, phages were passaged 8 times (one every two days) on the ancestral bacteria, by isolating phages (as described above) and transferring 60 µl of the phage solution with 60 µl of the corresponding ancestral bacterial culture to fresh microcosms with 6 ml of KB.

Every two transfers stocks were frozen for subsequent analysis at -80ºC (160 µl culture + 40 µl glycerol) (Poullain et al. 2008).

Efficiency Assays:

To estimate how evolutionary history affected phage efficiency (here measured as the density of phage particles that kill target bacteria) on different bacterial populations, PfUs were counted at three different time points of the experiment.

Phages from all populations were isolated (as previously described) at transfers 1, 4 and 8, and plated on bacterial lawns of naive bacteria (WT N_b and mutS N_b) and on allopatric bacteria coevolved with phages through four transfers (WT C_b and mutS C_b). These allopatric bacteria were coevolved in a preliminary experiment (described in Annex B). Both types of assay were performed on WT and mutS strains.

Coevolved bacteria for the assays (WT C_b and mutS C_b) were isolated by transferring a frozen crystal of each replicate (6 of WT and 6 of mutS) into 6ml KB and leaving it at 28ºC and 200 rpm overnight. Each of the resultant 12 microcosms was plated and bacterial colonies were grown for 48h at 28ºC. 10 individual bacterial colonies of each of the 12 coevolved populations (6 WT and 6 mutS) were then arbitrarily chosen and used to start a new 6ml overnight of that population.

Ancestral bacterial populations (WT N_b and mutS N_b) were obtained by starting overnights of naive ancestral WT and mutS populations (by transferring a frozen crystal of each population into 6ml KB) and plating the samples on KB agar. Samples were then grown for 48h at 28ºC. 120 individual bacterial colonies (60 for WT and 60 for mutS bacteria) were then arbitrarily chosen and partitioned into groups of 10 colonies. Each group was then used to start one of 12 new overnights (6 WT and 6 mutS).

Each of the above stocks (6 WT C_b, 6 mutS C_b, 6 WT N_b and 6 mutS N_b) was then used to make a bacterial agar lawn (5% of bacterial culture) on which the phage populations were rapidly inoculated: drops of 3 dilutions of each phage sample (all dilutions prepared from the same vial) were put in one of the 6 lawns of each bacterial population (WT C_b, mutS C_b, WT N_b and mutS N_b), so that each phage population was tested on each type of bacterial lawn. Lawns were kept at room temperature (23ºC) overnight and PfUs were counted the next day.

Statistical Analyses:

Analysis was performed for two response variables. The first was phage yield ($\log_{10}(n+1)$; n=number of phages); this measure likely reflects the capacity of the phage to infect bacterial cells and therefore our measure of interest, phage efficiency. However, as mentioned above, the phage aliquots added onto the bacterial lawns in the assay were not standardized to obtain one single phage concentration. Therefore it is possible that differences in phage efficiency were confounded with phage dose effects (for example, the observed increase in phage numbers with time may reflect an increase in efficiency of attack per phage particle and/or an increase in phage particle numbers).

To correct for phage dose, a model with a second response variable was analyzed, expressing phage yield on coevolved bacteria or on mutS bacteria relative to the phage yield on naive WT bacteria. Relative yield was calculated as a ratio, by dividing all (log-transformed) yields of a given phage by its yield on the naive WT bacteria (WT N_b; see Table 1 for symbol descriptions). In other words, this response variable can be considered as the per capita efficiency of a given phage on a given bacterial type (WT C_b, mutS N_b or mutS C_b). Previous work has compared (evolved) phage populations size by growing them on a susceptible (ancestral) bacterial genotype (Bull et al. 2000, Poullain et al. 2008. Note, however, that this technique assumes (i) that the ancestral bacteria are entirely susceptible to the phage and therefore phage counts most accurately reflect the total number of phage in an aliquot; (ii) that phages from later time points have fully retained their capacity to attack the ancestral bacteria.

Statistical models (mixed-design ANOVAs) analyzed effects of selection regime (coevolved or evolved; hereafter referred to as C/E) and bacterial genetic diversity (WT or mutS; hereafter referred to as WT/mutS) as factors. The interaction of these factors (C/E*WT/mutS) designates the evolutionary history of the phages because it crosses the selection process that the phages were exposed to with the type of bacteria with which they were selected. The models further contained time point (t1, t4, t8) and the type of bacteria used in the assays (assay B) as factors and phage selection line identity

as a random factor. Higher-order interaction effects were removed when non-significant (backward model simplification) to obtain a minimal adequate model.

Some WT N_b values from the first serial transfer were missing due to an error in the phage dilutions used for counting PfUs. This is because phage numbers were lower than expected (due to over dilution) and in some populations no PfUs could be counted; in all the populations in which they could be counted PfU numbers were very low. Since the dilutions would only allow us to count numbers above 500 000 phage particles, when we had 0 PfUs we assumed that the number of phage particles was lower than 500 000 and higher than or equal to 0. For such cases an intermediate value (250 000) was used as an "adjustment" (adj) on the statistical analysis.

Table 1 - Description of symbols.

WT C_p – Phage coevolved with WT bacteria

WT E_p – Phage evolved with WT bacteria

mutS C_p – Phage coevolved with mutS bacteria

mutS E_p – Phage evolved with mutS bacteria

WT C_b – WT bacteria that had been coevolved with phage until t4

mutS C_b – mutS bacteria that had been coevolved with phage until t4

WT N_b – Naive WT bacteria

mutS N_b – Naive mutS bacteria

Results

Analysis of phage yield indicates significant differences between coevolved or evolved phage selection regimes, among different time points (t1, t4 and t8) and among assay bacteria. Significant interactions of assay bacteria with time and the selection regime were also obtained (Table 2; R^2 =0.81).

It was found that all coevolved WT C_b bacteria were resistant to phages and that the coevolved mutS C_b bacteria were more resistant than the naive bacteria, but less than the coevolved WT C_b bacteria for phages at time points t4 and t8 (Fig. 1a, 1b), even though for t1 phages mutS C_b were also completely resistant (there is a strong bimodal pattern – phages either perform very well or not at all). On naive bacteria both coevolved and evolved phages produce similar yields (Fig. 1c, 1d). Phage's yields increased with time in all assays, but coevolved phages tended to have a higher yield, especially due to the low performance of the evolved mutS E_p phages (Fig. 1a, 1c, 1d and Suppl. Figure 1 in Annex A).

Table 2 - Effects of the phage selection regime (C/E), time point, assay bacteria (assayB) and the interactions of these factors on (log-transformed) phage yield (R^2=0.81) (mixed-design ANOVA). The Den. column indicates the denominator degrees of freedom for the F tests.

Source	DF	DF Den.	F	P
C/E	1	22	6.1044	0.0217*
Time	2	250	31.4588	<.0001*
assayB	3	250	148.7834	<.0001*
time*assayB	6	250	8.6914	<.0001*
C/E*assayB	3	250	4.1987	0.0064*

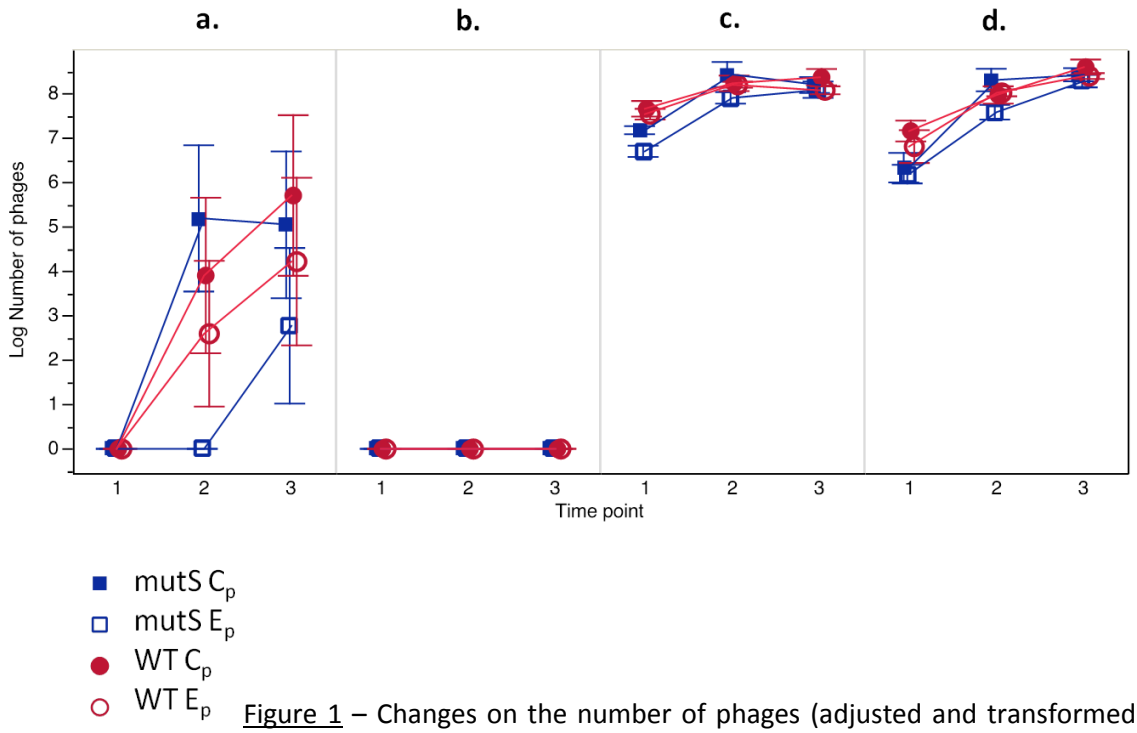

Figure 1 – Changes on the number of phages (adjusted and transformed data) on each population (mutS C_p, mutS E_p, WT C_p, WT E_p), per time point (1 = t1; 2 = t4; 3 = t8), on each type of bacterial lawn – **a.** mutS C_b; **b.** WT C_b; **c.** mutS N_b; **d.** WT N_b.

When standardizing the data for the phage yield on naive wild type lawns, the same significant effects as for the uncorrected data were obtained, except that (as expected) the levels of significance of some of the effects change, notably the effect of the type of phage passage (evolution vs. coevolution) goes from statistical significance to marginal significance (Table 3; $R^2 = 0.82$).

Figure 2c shows a negative slope on the mutS N_b bacteria. This indicates that the efficiency of the phage populations suffered a higher increase towards the WT N_b bacteria than towards mutS N_b bacteria, even though t1 phages were more efficient on mutS N_b bacteria than on WT N_b bacteria (ratio > 1). On Suppl. figure 2c (Annex A) it is clear that the ratios of the coevolved and evolved phage populations are overlapped.

Table 3 – Effects of the phage selection regime (C/E), time point, assay bacteria (assayB) and the interactions of these factors on the relative (log-transformed) phage yield, standardized by the yield on naive WT bacteria (R^2=0.82) (mixed-design ANOVA). The Den. column indicates the denominator degrees of freedom for the F tests.

Source	DF	DF Den.	F	P
C/E	1	22	3.8476	0.0626
Time	2	182	6.3134	0.0022*
assayB	2	182	196.3405	<.0001*
time*assayB	4	182	15.1095	<.0001*
C/E*assayB	2	182	5.2335	0.0062*

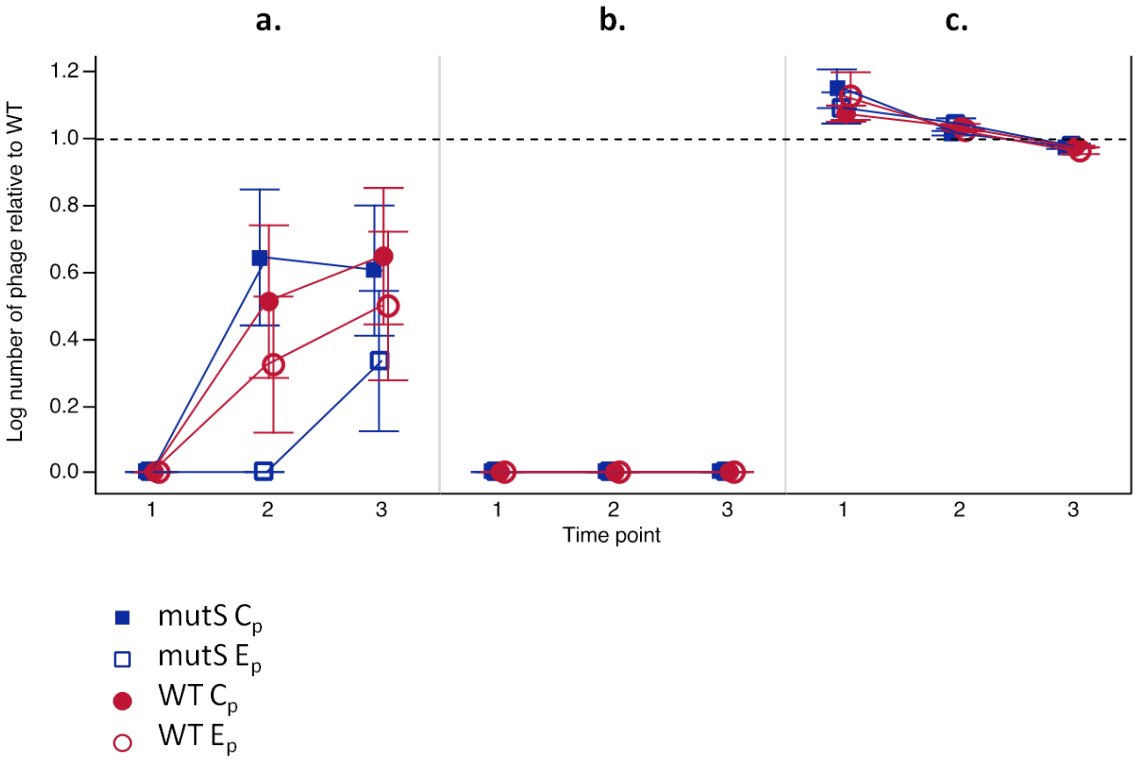

Figure 2 – Changes on the number of phages (adjusted and transformed data) on each population (mutS C_p, mutS E_p, WT C_p, WT E_p), per time point (1 = t1; 2 = t4; 3 = t8), for the ratio of – **a.** mutS C_b, **b.** WT C_b and **c.** mutS N_b by the WT N_b.

Discussion

Phage therapy is a viable alternative to antibiotic treatments in treatments against certain bacterial infections (Abedon 2009). Despite many promising advantages, there are still numerous challenges that will require additional scientific investigation (Loc-Carrillo & Abedon 2011, Chanishvili et al. 2001, Dublanchet & Patey 2011).

In this study, it was tested how the selection regime (evolution versus coevolution on wild-type (WT) versus mutator (mutS) bacteria) may alter the efficiency of phages. Efficiency is quantified as the density of phage particles that kill target bacteria. In a comparable study, Poullain and colleagues (2008) verified that phage evolution resulted in adaptation through higher growth rates, whilst coevolved phages increased their infectivity range. By comparing the efficiency of different treatments administered to phage populations and measured on a reference bacterial population, this study has contributed to the development of an experimental protocol that increases phage efficiency, with the objective of controlling bacterial pathogens.

It can be observed that all WT coevolved bacteria were resistant to phages, whether these were from the past (three transfers previous), present or future (eight transfers subsequent) (Fig 1b). The bacteria used in the assays were not sympatric with the phage populations, so this could mean that these coevolved bacteria became completely resistant to allopatric phages. Evolved resistance to phages often occurs through changes in cell surface receptor molecules to which phages attach (Bohannan & Lenski 2000, Lenski 1988, Hofnung et al. 1976, Mizoguchi et al. 2003, Dupont et al. 2004). Should such receptors be involved in cell metabolism, then a change in their configuration could impair this function and constitute a cost associated with the adaptation (Bohannan & Lenski 2000, Lenski 1988, Lennon et al. 2007). Resistance to all phages as observed in this study could result from the complete loss of the phage's binding site (Lenski 1984, Lenski & Levin 1985). That this could have been what happened to the coevolved WT bacteria in this study, is suggested by the lower densities of some populations compared with control bacteria (data not shown). The putative costs could be confirmed by competing these bacteria with naive, or other not completely resistant bacteria, with the prediction that treatment bacteria should be outcompeted. The fact that completely resistant bacteria are outcompeted by bacteria resistant only to contemporary phages is what allows for directional coevolution with phages to continue (Buckling & Rainey 2002).

In contrast to WT bacteria, mutS coevolved bacteria were only completely resistant to phages from the past, and phage efficiency increased when their evolutionary history included a larger number of generations coevolving with other (allopatric) bacterial populations (Figure 1a) (Poullain et al. 2008, Buckling & Rainey

2002). Also, all phage populations (whether coevolved or evolved) proved to be more efficient against naive bacteria than they were against the coevolved bacteria (Figure 1) (Poullain et al. 2008, Buckling & Rainey 2002), but the coevolved phages performed better against the coevolved bacteria that did evolved phages (Suppl. figure 1). This could be due to the possibility that evolved phages suffered a cost associated with their adaptations when confronted with coevolved hosts (Poullain et al. 2008). Previous studies suggest that phage costs could have pleiotropic origins (Ebert 1998, Crill et al. 2000, Duffy et al. 2006) or be a consequence of physical constrains in genome size (if higher efficiency is linked to the acquisition of new genes) (De Paepe & Taddei 2006). The fact that the coevolved phages were tested on allopatric bacterial populations ensured that their efficiency was not overestimated due to a better performance on their sympatric bacteria.

In comparison with coevolved WT bacteria, coevolved mutS bacteria were therefore more sensitive to all four phage populations (Figure 1a and b). These results are in part consistent with those of Buckling et al. 2005, who suggested that phages coevolving with bacteria with high mutation loads have an advantage over these bacteria. However, no significant difference between the phages selected on WT and mutS bacteria was found. According to Pal et al. 2007, coevolution with phages can lead to an increase of the bacterial mutation rates (it happened to 25% of their coevolving populations), since mutators may be indirectly favored due to hitch-hiking with alleles that give resistance to the coevolving phage populations. This could mean that some of the WT and mutS bacterial populations in which the phages of this study were selected may not have been as different as expected, even though, since only 8 transfers were conducted, mutators in the WT populations probably did not have time to increase substantially in frequency.

When confronted with naive bacteria, phage infectivity increased mostly until transfer 4 and far less between transfers 4 and 8, particularly on mutS naive bacteria (Figure 1c and d). This could be due to phage infectivity to ancestral bacteria being close to its peak after only a small number of transfers (for evolved phages) or, in the case of coevolved phage populations, could be due to the existence of costs of specialization (Poullain et al. 2008). It can also be observed a large variation in the performance of the phage population replicates on mutS coevolved bacteria (Figure 1a). This could be due to different replicates of coevolved bacteria (and phage) following divergent coevolutionary trajectories (Buckling and Rainey 2002). The extent to which different specific selected mutations may have been involved in this variation, or how much it was influenced by chance events and measurement error, was not determined.

Phage populations showed no significant differences in efficiency when confronted with WT compared to mutS naive bacteria (Figure 1c and d). While some

bacterial mutations can be beneficial, the majority probably have deleterious effects (Sturtevant 1937, de Visser 2002). Laboratory experiments have shown that high mutation rates may sometimes result in very rapid evolution of resistance (Chopra et al. 2003, Perron et al. 2010) and that mutators are more likely to evolve and conserve resistance to antibiotics in the presence of bacteriophages (Escobar-Páramo et al. 2012, Zhang & Buckling 2012), although with large fitness costs (Zhang & Buckling 2012), that sometimes they are able to compensate (Perron et al. 2010).

As mentioned above (see Statistical Analyses), there is a possibility that the differences in phage efficiency were confounded with phage dose effects. In an effort to obtain the per capita efficiency of a given phage on a given bacterial type, models that expressed phage yield on coevolved bacteria or on mutS bacteria relative to the phage yield on naive WT bacteria were analyzed. This method assumes both that the ancestral bacteria are entirely susceptible to the phages, and therefore phage counts accurately reflect the total number of phages in an aliquot, and that phages from later time points have fully retained their capacity to attack the ancestral bacteria. However, it is possible that there is a trade-off between the coevolved phage's higher efficiency and their capacity of infecting the ancestral bacteria (a cost), so phage densities could be underestimated (as referred by Poullain et al. 2008). Nevertheless, only overall differences among phages or among time points could be due to dose effects. For example, the differential performances of phages on different assay bacteria (C/E*assayB) are unlikely to result from dose effects because, for a given phage population, the same number of particles is plated on the 4 different assay bacteria, and therefore differential performance is not due to dose effects. The fact that in the first transfer phage populations were more efficient on mutS naive bacteria than on WT naive bacteria (ratio over 1) (Figure 2c) suggests that, indeed, phage densities could be being underestimated on the WT naive bacteria or that, at this time point, mutS naive bacteria were simply more sensitive to phages (similar to coevolved bacteria).

Hence, this study suggests that coevolved phages are more efficient in killing bacteria than evolved ones. However, more studies are needed to correct for the potential dose effect. For example, other phage counting techniques (like fluorescent or electronic microscopy) (Nie et al. 1995, Børsheim et al. 1990) could be used simultaneously with plating in order to determine true phage numbers. Also, streaking assays could be used to test local adaptation of phage and bacterial populations and costs (Buckling & Rainey 2002, Poullain et al. 2008). A future approach could also consist of confronting coevolved/evolved phage populations with biofilms, to observe their performance in such situation. Bacteriophages have already been observed to cause reduction of biofilms of *P. fluorescens* (Sillankorva et al. 2004) and of antibiotic resistant biofilms of *P. aeruginosa* (Pires et al. 2011).

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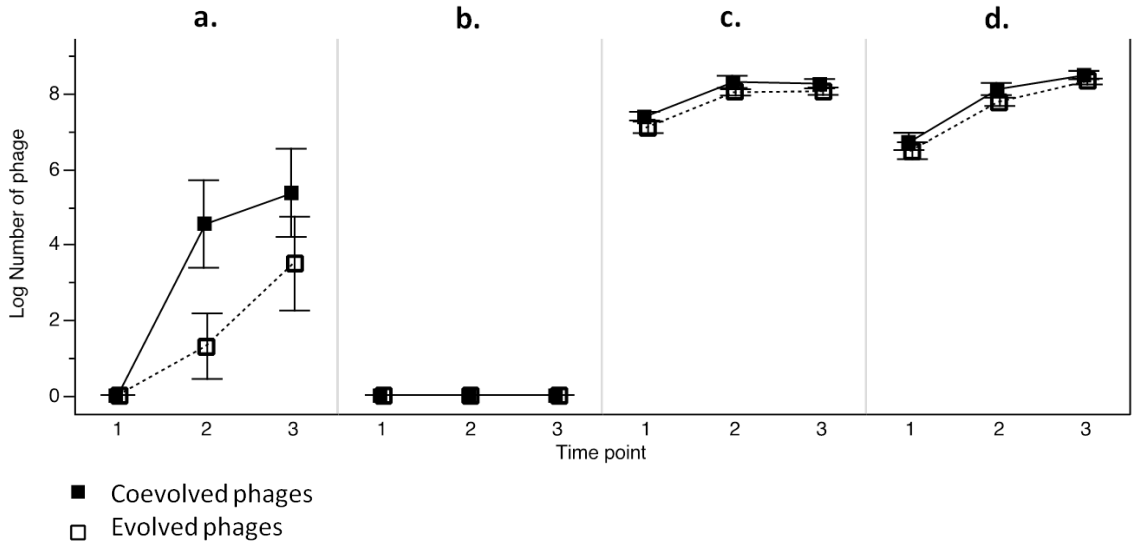
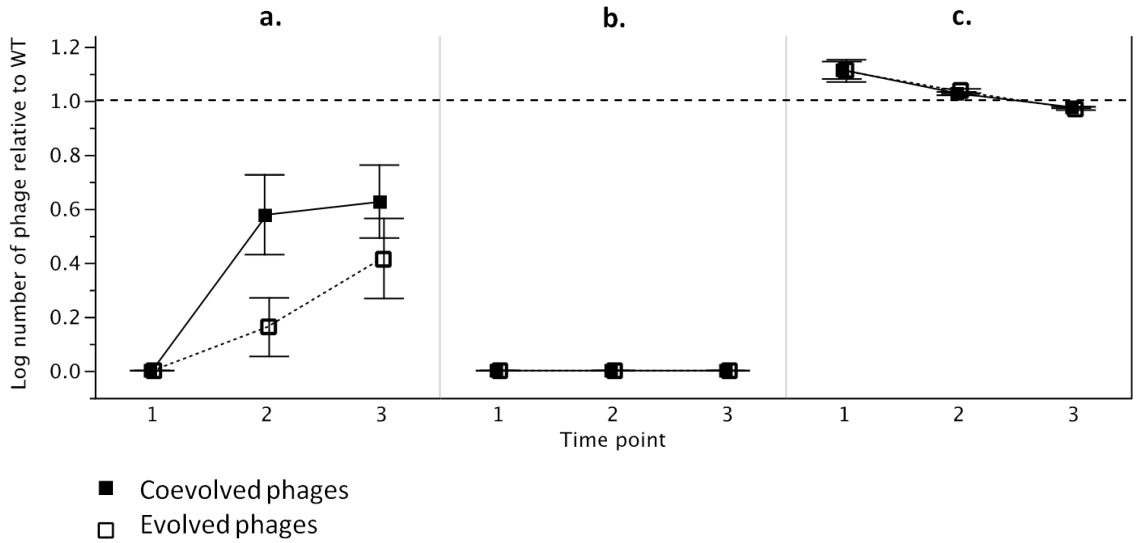
Annexes

Annex A – Main experiment supplementary figures



Suppl. figure 1 – Changes on the number of phages (adjusted and transformed data) on each selection regime (C, E), per time point (1 = t1; 2 = t4; 3 = t8), on each type of bacterial lawn – **a.** mutS C_b; **b.** WT C_b; **c.** mutS N_b; **d.** WT N.



Suppl. figure 2 – Changes on the number of phages (adjusted and transformed data) on each selection regime (C, E), per time point (1 = t1; 2 = t4; 3 = t8), for the ratio of – **a.** mutS C_b, **b.** WT C_b and **c.** mutS N_b by the WT N_b.

Annex B – Preliminary experiment

- Protocol

Sections *System*, *Culture Conditions* and *Selection Experiment* are equal for this experiment and the main one (see Table 1 for description of symbols).

Phages of all populations were isolated from transfers 1, 4 and 8 and plated on bacterial lawns of WT and mutS naive bacteria (WT N_b and mutS N_b, respectively). The number of phage plates (PfU) was counted in three different dilutions (10^{-4}, 10^{-5} and 10^{-6}) and the number of phages was estimated.

Stocks with coevolved bacteria from this experiment were used to prepare the bacterial lawns of coevolved bacteria (WT C_b and mutS C_b) of the main experiment (see the section *Efficiency Assays* of the main text).

Analysis was performed for two response variables. The first was phage yield ($\log_{10}(n+1)$; n=number of phages); this measure likely reflects the capacity of the phage to infect bacterial cells and therefore our measure of interest, phage efficiency.

Like in the main experiment, to correct for phage dose, a model with a second response variable, expressing phage yield on naive mutS bacteria relative to the phage yield on naive WT bacteria, was analyzed. Relative yield was calculated as a ratio, by dividing (log-transformed) yields of a given phage by its yield on the naive WT bacteria (WT N_b; see Table 1 for symbol descriptions). In other words, this response variable can be considered as the per capita efficiency of a given phage on mutS N_b bacteria.

Statistical models (mixed-design ANOVAs) analyzed effects of selection regime (coevolved or evolved; hereafter referred to as C/E) and bacterial genetic diversity (WT or mutS; hereafter referred to as WT/mutS) as factors. The interaction of these factors (C/E*WT/mutS) designates the evolutionary history of the phages because it crosses the selection process that the phages were exposed to with the type of bacteria with which they were selected. The models further contained time point (t1, t4, t8) as a factor and phage selection line identity as a random factor. Higher-order interaction effects were removed when non-significant (backward model simplification) to obtain a minimal adequate model.

Some values from t1 were missing.

- Results

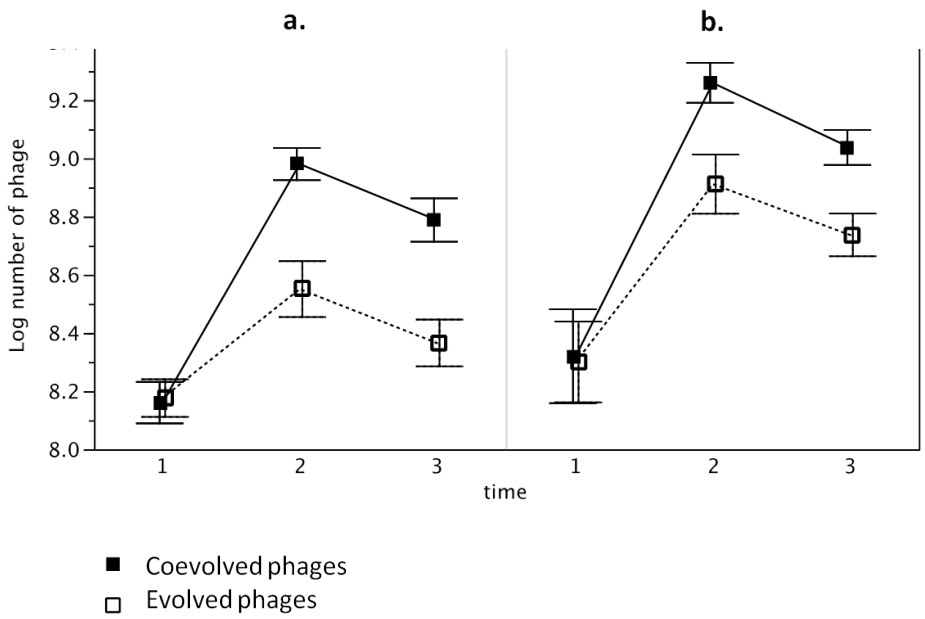
Analysis of phage yield indicates that the phages behave significantly different when on different time points and when they are plated on different bacterial lawns. The interactions between the strain in which the phages were selected and the selection regime with time (WT/mutS*time, C/E*time), as the interaction between these three factors (WT/mutS*C/E*time), are also significant (Suppl. table 1; R^2 =0.82).

On Suppl. figure 3 it can be seen that, for t4 and t8, Coevolved phages are always on higher numbers than Evolved phages, whether in mutS or WT naive bacteria.

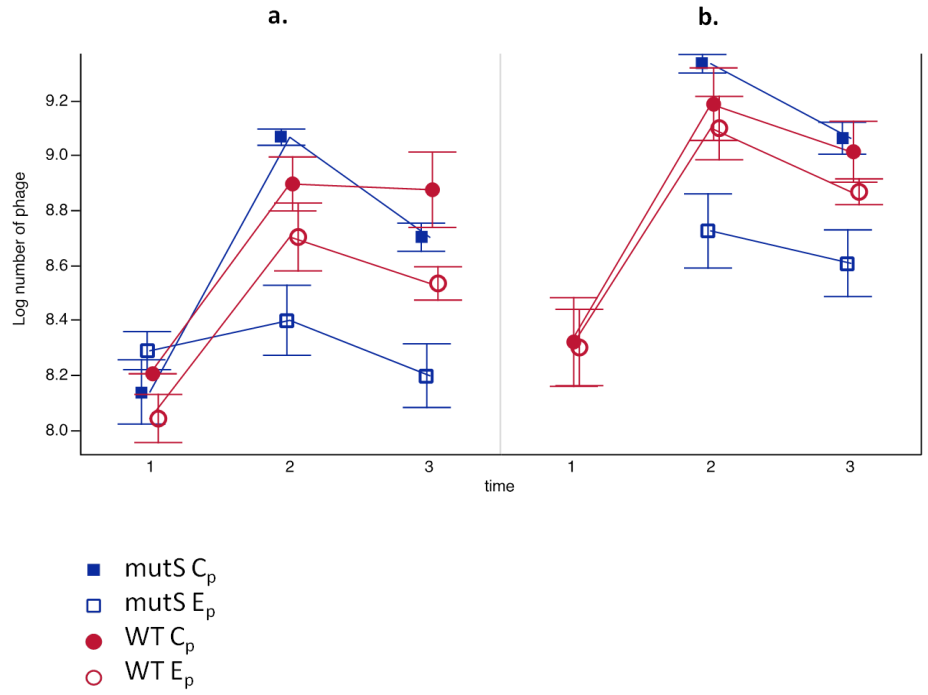
On WT naive bacteria there are two phage populations (mutS C_p and mutS E_p) in which there was not enough data on t1 to compare this time point with the other two (which may also contribute to the fact that time is highly significant) (Suppl. Figure 4b).

Suppl. table 1 - Effects of the bacterial strain in which the phages were selected (WT/mutS), phage selection regime (C/E), time point, assay bacteria (assayB) and the interactions of these factors on (log-transformed) phage yield (R^2=0.82) (mixed-design ANOVA). The Den. column indicates the denominator degrees of freedom for the F tests.

Source	DF	DF Den.	F	P
WT/mutS	1	92.46	2.3311	0.1302
C/E	1	89.61	0.4409	0.5084
WT/mutS*C/E	1	89.61	0.5869	0.4456
Time	2	90.72	61.8946	<.0001*
assayB	1	88.98	58.9643	<.0001*
WT/mutS*time	2	90.68	4.4730	0.0140*
C/E*time	2	90.76	8.4244	0.0004*
WT/mutS*C/E*time	2	90.76	4.2405	0.0174*



Suppl. figure 3 – Changes on the number of phages (transformed data) on each selection regime (C, E), per time point (1 = t1; 2 = t4; 3 = t8), on each type of bacterial lawn – **a.** mutS N_b, **b.** WT N_b.



Suppl. figure 4 - Changes on the number of phages (transformed data) of each population (mutS C_p, mutS E_p, WT C_p, WT E_p) per time point (1 = t1; 2 = t4; 3 = t8) and on each type of bacterial lawn – **a.** mutS N_b, **b.** WT N_b.

When standardizing the data for the phage yield on naive WT lawns, it was impossible to test the three time points at the same time due to the missing values from t1. Here, two separated analyses were made, one for t1 and another for t4+t8.

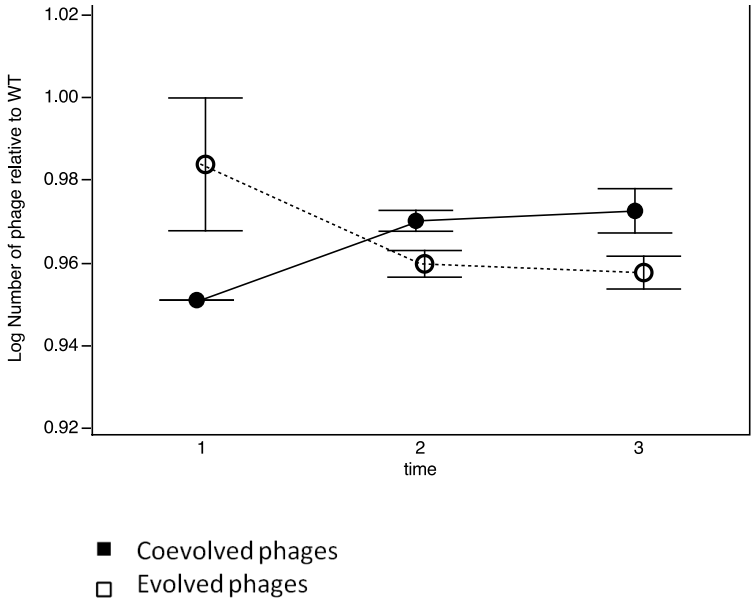
When considering only t1, there were no significant effects. When considering t4+t8 both the selection regime of the phage and the interaction of the strain in which phages were selected with time gain significance (Suppl. table 2).

On Suppl. figure 5 it can be seen that coevolved phages have a general positive slope, which means that these phages increased more in efficiency on mutS than on WT naive bacteria. Evolved phages have a general negative slope, which means that these phages increased more in efficiency on WT than on mutS bacteria. Both ratios are always under 1, which means that the WT naive bacteria were more sensitive to the phages than the mutS bacteria.

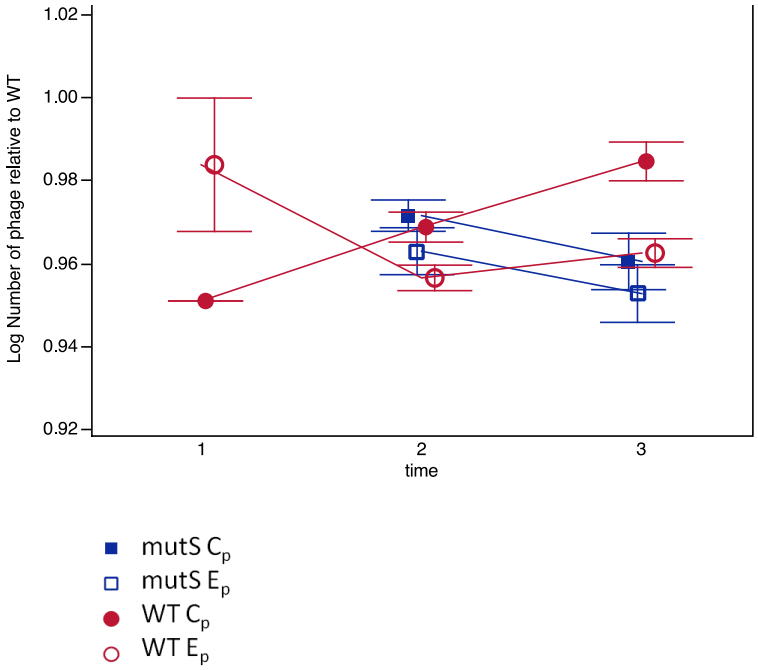
The slopes of each individual phage population can be seen in Suppl. figure 6.

Suppl. table 2 - Effects of the bacterial strain in which the phages were selected (WT/mutS), phage selection regime (C/E), time point and the interactions of these factors on the relative (log-transformed) phage yield, standardized by the yield on naive WT bacteria, for t1 (R^2=0.22) and t4+t8 (R^2=0.50) (mixed-design ANOVAs). The Den. column indicates the denominator degrees of freedom for the F tests.

	Source	DF	DF Den.	F	P
t1	C/E	1	3	0.84	0.4282
t4+t8	time	1	22	0.0019	0.9652
	WT/mutS	1	42.17	0.8633	0.3581
	C/E	1	21	11.7411	0.0025*
	WT/mutS*time	1	22	10.7290	0.0035*



Suppl. figure 5 – Changes on the number of phages (transformed data) on each selection regime (C, E), per time point (1 = t1; 2 = t4; 3 = t8), for the ratio of mutS N_b by WT N_b.



Suppl. figure 6 – Changes on the number of phages (transformed data) of each population (mutS C_p, mutS E_p, WT C_p, WT E_p), per time point (1 = t1; 2 = t4; 3 = t8), for the ratio of mutS N_b by WT N_b.

- Discussion

It can be observed that, like in the main experiment, coevolved phages have a higher yield (Suppl. Figure 3), even though in this case they did not perform significantly different from the evolved phages (Suppl. table 1). In this experiment, phage populations confronted with ancestral bacteria tended to reach their maximum at transfer 4, then decreasing in density until transfer 8 (Suppl. figure 4). Again, this can result from phages quickly reaching their infectivity peak or from the aquisition of specialization costs (Poullain et al. 2008). These phage populations were also bigger than the ones of the main experiment (Figure 1 and Suppl. figure 4), and here the ratios are always under 1 (Suppl. figure 5 and 6), which means that these naive WT bacteria were always more sensitive to all phage populations than the naive mutS bacteria, whether there was an underestimation of phage numbers on the WT bacteria or not.

Hence, both studies suggest that coevolved phages are more efficient in killing bacteria than evolved ones. The differences between both experiments may be due to, for example, slightly different initial populations of phages and bacteria (sampling effects), time effects (since both experiments were not made at the same time) and differences on the medium (KB constitution can vary).